

The *lin-15* Locus Encodes Two Negative Regulators of *Caenorhabditis elegans* Vulval Development

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During *Caenorhabditis elegans* vulval development, an inductive signal from the anchor cell stimulates three of the six vulval precursor cells (VPCs) to adopt vulval rather than nonvulval epidermal fates. Genes necessary for this induction include the *lin-3* growth factor, the *let-23* receptor tyrosine kinase, and *let-60 ras*. *lin-15* is a negative regulator of this inductive pathway. In *lin-15* mutant animals, all six VPCs adopt vulval fates, even in the absence of inductive signal. Previous genetic studies suggested that *lin-15* is a complex locus with two independently mutable activities, A and B. We have cloned the *lin-15* locus by germline transformation and find that it encodes two nonoverlapping transcripts that are transcribed in the same direction. The downstream transcript encodes the *lin-15A* function; the upstream transcript encodes the *lin-15B* function. The predicted *lin-15A* and *lin-15B* proteins are novel and hydrophilic. We have identified a molecular null allele of *lin-15* and have used it to analyze the role of *lin-15* in the signaling pathway. We find that *lin-15* acts upstream of *let-23* and in parallel to the inductive signal.

INTRODUCTION

Intercellular signaling is a common mechanism used by multicellular organisms to specify cell fate during development, and receptor tyrosine kinases (RTKs)¹ (reviewed by Fantl *et al.*, 1993) represent one important class of signaling molecules. In general, the extracellular domain of an RTK binds an intercellular signal and regulates an intracellular tyrosine kinase activity. Upon ligand stimulation, RTKs associate with substrates and other signal transducers. RTKs involved in development include the insulin family RTK *sevenless* required for *Drosophila* photoreceptor development (Hafen *et al.*, 1987), the platelet-derived growth factor (PDGF) family RTK *torso* involved in *Drosophila* terminal development (Sprenger *et al.*, 1989), the *Drosophila* epidermal growth factor (EGF) receptor homologue DER involved in *Drosophila* dorsal/ventral patterning, ommatidial patterning in eye imaginal discs, and neuronal fate (Price *et al.*, 1989; Schejter and Shilo, 1989), the *c-kit* RTK encoded

by the W-locus utilized in mouse hematopoiesis and spermatogenesis (Chabot *et al.*, 1988), and the fibroblast growth factor receptor used in *Xenopus* mesoderm induction (Amaya *et al.*, 1991).

RTK activity is normally modulated by reception of ligand (Massagué and Pandiella, 1993). Thus, both ligand expression and localization are important for regulating RTK activity. Control of the expression of the RTKs themselves is another regulatory mechanism; overexpression of the EGF-RTK is seen in human solid tumors as well as tumor cell lines, although it is unclear whether this is a cause or an effect (Cowley *et al.*, 1984; Lin *et al.*, 1984; Merlino *et al.*, 1984; Ullrich *et al.*, 1984; Derynck *et al.*, 1987). Furthermore, downregulation of RTK-mediated signaling through phosphorylation of defined sites of the receptor C-terminus, the activities of phosphatases, and the activity of protein kinase C also occurs (reviewed by Carpenter and Wahl, 1990). Negative regulation of the EGF-RTK has been proposed to occur through Müllerian Inhibitory Substance (Cigarro *et al.*, 1989), a transforming growth factor- β homologue (Cate *et al.*, 1986), although this inhibition may be indirect. Other events, such as ligand-mediated internalization and receptor dimerization, also define steps where potential regulation of RTK activity could occur.

¹ Abbreviations used: AC, anchor cell; Berg, Bergerac; Bris, Bristol; EMS, ethylmethanesulfonate; Muv, multivulva; PCR, polymerase chain reaction; Rol, rolling; RTK, receptor tyrosine kinase; VPC, vulval precursor cell; Vul, vulvaless.

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During *Caenorhabditis elegans* vulval development, a signal is sent from the anchor cell (AC) of the hermaphrodite gonad to the vulval precursor cells (VPCs), which are P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p, in the ventral epidermis (Kimble, 1981). This inductive signal causes three of the six VPCs to undergo vulval development, whereas the other three cells undergo nonvulval epidermal development. The three VPCs adopting vulval fates do so in a stereotyped pattern. P6.p, the VPC closest to the anchor cell adopts a 1° fate; the neighboring P5.p and P7.p adopt 2° fates. Removal of the inductive signal causes all VPCs to adopt nonvulval epidermal fates (Kimble, 1981; Sulston and White, 1980). Cells that adopt vulval fates undergo three rounds of cell divisions, characterized by stereotyped cell division patterns and morphogenetic movements; cells that adopt nonvulval epidermal fates divide at most only once (Sulston and Horvitz, 1977). The direct observation of cell division patterns provides a straightforward assay of VPC fate.

The inductive signal provided by the AC is an EGF-like growth factor encoded by *lin-3* (Hill and Sternberg, 1992; Hill, Katz, and Sternberg, unpublished data). *let-23*, the candidate receptor for the signal, encodes an EGF-like RTK (Aroian *et al.*, 1990). Several molecules downstream of *let-23* have been identified, including *sem-5*, a SH2/SH3 adaptor protein (Clark *et al.*, 1992); *let-60*, a *ras* homologue (Han and Sternberg, 1990); and *lin-45*, a *raf* homologue (Han *et al.*, 1993) (see Figure 1). Reduction of function mutations in any of these genes result in a vulvaless (Vul) phenotype, where all six VPCs adopt nonvulval epidermal fates.

Loss of function mutations in the *lin-15* locus result in an opposite phenotype. All six VPCs adopt vulval fates, leading to a multivulva (Muv) phenotype (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). Thus, *lin-15* is a negative regulator of vulval differentiation. Genetic mosaic analysis indicates that *lin-15* acts in cells other than the AC and the VPCs (i.e., nonautonomously) (Herman and Hedgecock, 1990). *lin-15* is not the only locus involved in this aspect of negative regulation. *lin-15* is a member of a set of negative regulators; appropriate combinations of mutations in these loci cause a Muv phenotype (Ferguson and Horvitz, 1989). This group of negative regulators was first defined by the genes *lin-8* and *lin-9* and now includes many other loci. *lin-8* and *lin-9* define class A and class B genes, respectively. Animals carrying a single mutation in either A or B genes are wild-type; only animals homozygous mutant for both an A and a B gene are Muv. *lin-15* is a complex locus; it is the only genetically identified locus in this pathway to have three kinds of alleles. The A and B alleles of *lin-15* have a wild-type vulval phenotype. There are also alleles that confer a Muv phenotype; we refer to these as AB alleles. We cloned the *lin-15* locus to study the basis for the two functions and to elucidate the role of *lin-15* in vulval differentiation.

MATERIALS AND METHODS

Strains and Culture Conditions

Nematodes were cultured as described (Brenner, 1974; Sulston and Hodgkin, 1988). All experiments were performed at 20°C unless otherwise noted. The following mutations and strains were used in this study: linkage group X: the *lin-15* alleles *n309*, *e1763*, *n1139*, *n765* (Ferguson and Horvitz, 1985), *n744*, *n767* (Ferguson and Horvitz, 1989), *n744sy212*, *n744sy211* (this study), *n767sy222* (Hill, personal communication), *sy197* (Lee, personal communication), *sup-10(n983)* (Greenwald and Horvitz, 1982), *mec-5(e1340)* (Chalfie and Sulston, 1981), *unc-3(e151)* (Brenner, 1974), *lin-8(n111)* (Ferguson and Horvitz, 1985), and *mnDp1(X;V)*; *mnDf4 (X)* (Meneely and Herman, 1979); linkage group III: *lin-36(n766)* (Ferguson and Horvitz, 1989); linkage group II: *let-23(sy97)/mnC1(dpy-10 unc-52)* (Herman, 1978; Aroian and Sternberg, 1991); the wild-type *C. elegans* strain N2 (Brenner, 1974), and the Bergerac (Berg) BO *C. elegans* strain (Emmons *et al.*, 1979). Genetic nomenclature follows Horvitz *et al.* (1979), except we designate the *lin-15* allele *n765* as "*lin-15ABⁿ(n765)*" because animals carrying this mutation display a Muv phenotype at 20°C, although the B function is restored at 15°C (Ferguson and Horvitz, 1985).

Physical Mapping of the *lin-15* Locus

We mapped the *lin-15* locus by constructing recombinants between the *C. elegans* Bristol (Bris) strain and the Berg strain, a *C. elegans* strain that contains many polymorphisms with respect to the Bris strain as described (Rose *et al.*, 1982). Because *lin-15* is X-linked and *C. elegans* males are XO, we allowed Bris N2 males to mate with Berg BO hermaphrodites. Berg/0 males from this cross were then allowed to mate with *lin-15ABⁿ(n765 Bris) sup-10(n983 Bris)* hermaphrodites, and their heterozygous cross-progeny hermaphrodites (*lin-15ABⁿ(n765 Bris) sup-10(n983 Bris)/lin-15(+ Berg) sup-10(+ Berg)*) were selected and grown at 25°C. Their progeny were screened for animals with the Sup-10(n983) phenotype but not the Muv phenotype; these were presumed to be of the genotype *lin-15ABⁿ(n765 Bris) sup-10(n983 Bris)/lin-15(+ Berg) sup-10(n983 Bris)*. These animals were allowed to self-fertilize, and their Sup-10 progeny that did not segregate Muv animals in the next generation were kept. Twenty recombinants were found. DNA from these animals was prepared, digested with restriction enzymes, Southern blotted, and probed with TU#W1723, a clone that identified a polymorphism from the *sup-10* contig (Driscoll and Chalfie, personal communication). Of the genomic DNA from the 20 recombinants, 13 displayed a Bris pattern, five displayed a Berg pattern, and two were ambiguous.

Germline-mediated Transformation by Microinjection

Microinjection was performed as described (Mello *et al.*, 1991), modified from Fire (1986). For all injection experiments, the plasmid pRF4, containing the *rol-6(su1006)* mutant gene (Mello *et al.*, 1991), was used as a transformation marker at a concentration of 50 ng/μl. Injected animals were placed on new plates at least once per day, and their progeny were examined one to four times per day. F1 rolling (Rol) animals were placed on individual plates as soon as they were identified and scored under a dissecting microscope for rescue of the Muv phenotype upon reaching adulthood. Their progeny were examined for the presence of Rol animals to determine whether the line was stable, and if stable, were checked for their Muv phenotype. All injected animals, F1 transformants, and transgenic lines were maintained at 20°C. Animals of the genotype *lin-15ABⁿ(n765)*, *lin-8A(n111)*; *lin-15B(n744)*, and *lin-36B(n766)*; *lin-15A(n767)* were injected, as described in Figure 2, Figure 8, and Table 1.

RNA Analysis

Mixed stage RNA was prepared from N2 animals using guanidinium thiocyanate and pelleted using CsTFA (Pharmacia, Piscataway, NJ).

Poly A⁺ selection was performed using a poly dT column (Pharmacia). Twice selected poly A⁺ RNA (7.5 µg) was separated on a 1% denaturing formaldehyde agarose gel, blotted onto Hybond-N (Amersham, Arlington Heights, IL), and probed with random-primed DNA labeled with ³²P, all according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). Exposures were done for 2 1/2 wk at -70°C with an intensifying screen on Kodak (Rochester, NY) XAR film.

Subclones and Southern blots

All subcloning, Southern blotting, and DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). *C. elegans* genomic DNA was prepared according to the methods of Andy Fire as described by Sulston and Hodgkin (1988).

cDNA Analysis

Two cDNA libraries were screened with the 6.8-kilobase (kb) *Bam*HI fragment of plasmid p68B3 (see Figure 1 for location) using standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992) because p68B3 hybridizes with both transcripts detected on a Northern blot (see Figure 2). One cDNA library was in λgt10 (kindly provided by S. Kim, Stanford University); the other was in λSHLX2 (kindly provided by C. Martin, Columbia University). Two cDNAs were obtained from the Kim library and six from the Martin library. Two noncross-hybridizing classes of cDNAs were detected, and both classes were represented in both libraries. These cDNAs were mapped to genomic DNA through Southern hybridization using probe synthesized from the cDNA clones against genomic clones digested with various restriction enzyme combinations and blotted on nylon membranes.

The composite cDNA for the upstream transcript appeared not to include the 5' end of the gene based on the apparent size of the transcript as detected on a Northern Blot (see Figure 3). Therefore, a third cDNA library in λZAP (kindly provided by B. Barstead, Oklahoma Medical Research Foundation) was probed with pBLH10, which contains a 1.3 kb-*Eco*RI fragment from base 3045 to base 4396, upstream of the most 5' cDNA sequence. Six cDNAs were obtained; three were identical clones (cF1, cD1, cF2). The four unique cDNAs were *lin-15* clones, as demonstrated by DNA sequencing. However, two of the four cDNAs (cF1, cD3) were fused to cDNAs from other genes, as evidenced by the presence of an *Eco*RI linker fused to a poly A⁺ tail in the middle of the clone. A third cDNA (cD2) ended at the 3' end of the *lin-15* upstream transcript but had a highly rearranged 5' end in previously sequenced DNA. The fourth cDNA (cL1) was an intact cDNA that started 848 basepair (bp) 5' of the genomic 3' end of the upstream transcript. Sequence from cL1 gave 286 bp more cDNA sequence from the previous 5' end. Sequence from the *lin-15* portion of cF1 gave 1110 bp more cDNA sequence upstream of that obtained from cL1. However, the cDNA sequence from cF1 was separated from the cDNA sequence inferred from cL1 by a 35 bp genomic gap that was later found to be transcribed, as described below.

Exon Predictions

Because the most 5' cDNA of the upstream transcript was not full length, we predicted potential exons by examining the genomic sequence. Because *C. elegans* introns have, on average, a 68% AT content (Fields, personal communication), the genomic region was plotted for its relative AT versus GC content. AT-rich regions were considered potential introns. The genomic DNA sequence was scanned by eye for potential splice donor and acceptor sites (Emmons, 1988) that would maintain the open reading frame, on the basis of our known cDNA sequence. Thus, the exons predicted had to fulfill three criteria. 1) The exon had to have an open reading frame that would splice in-frame to the next exon. 2) The exon had to have splice donor and acceptor sites of reasonable consensus. 3) The exon had to have a GC content of >35%.

To confirm exon predictions, we used 4.5 µg of once-selected poly A⁺ RNA to synthesize cDNA using primer m1sq (CGG GTA ACG

TAG TTG TAG), primer w2sq (GGA AAC GAT ATC CCA TTG AG), primer 18BJ7B (GCG GTT TAG ATG ATG TGC TG), and primer 20J7A (GCG ATA AAT ACA GCC CAA C). The polymerase chain reaction (PCR) was performed on one-tenth of the synthesized cDNA using primer bF (ACA GCA CGA CTT ACA TCA AAC CCG GCA TCA), primer wF (GGC CGT TGC CAC CGA GAC CAG CCA ATG TGC C), primer mR (CGT TTG ACA ACT GCA CTC TCT TCA ACC AGC), primer wR (GTT GCG AAT TGA CAT GAT GGC TGG CAC AAC), and primer xR (CGG TTG CAA GCT CTG CGC TTC TGG AAT TCC) to confirm exon predictions. PCR was performed using Pfu DNA Polymerase (Stratagene, La Jolla, CA) using the following conditions: 95°C, 1 min; 60°C, 1 min; 72°C, 1 min 30 sec, for 26 cycles. Band of predicted sizes were obtained using combinations of primer bF with primer mR, primer bF with primer wR, and primer wF with primer xR. The bands obtained were cloned and sequenced according to standard methods.

Identifying Trans-splicing to SL2

cDNA was made from 30 µg of N2 RNA using primers A4-RA6 (GAG ATA CTC GAT GAT CCA GC) and D32R (CGG TTT GAC AGT TGG GAG). PCR was performed using primers SL2 (GGT TTT AAC CCA GTT ACT CAA G) and A4-VR6 (GAT GAG TTG AAT GAG TTC TTG CTT ATC AGC), using one-fifth of the cDNA synthesized. Primer SL2 is the exact sequence of the SL2 trans-splice leader (Huang and Hirsh, 1989). One-tenth of the PCR reaction was separated on a 0.8% Tris-Borate-EDTA (TBE) gel and blotted onto Hybond-N (Amersham). The blot was probed with random primed B11c, a cDNA of the downstream transcript obtained from the Martin library, to establish that the 1.2-kb band visualized with ethidium bromide staining was indeed a *lin-15* clone. Three different thermostable polymerases, Taq (Cetus, Berkeley, CA), Vent (New England Biolabs, Beverly, MA), and Pfu (Stratagene) were used in three independent PCR reactions to minimize polymerase artifacts. The 1.2-kb bands from the three different reactions were isolated from a low melting point TBE agarose gel, cloned, and sequenced to confirm their identity as *lin-15* clones.

Sequencing

Double strand sequencing of all genomic and cDNA clones was performed using a combination of primer walking, subcloning, and γδ transposon sequencing as described (Sambrook *et al.*, 1989; Strathmann *et al.*, 1991; Ausubel *et al.*, 1992). Sequencing reactions were performed using Sequenase version 2.0 and other reagents from United States Biochemical (Cleveland, OH). Sequence was compiled using the International Biotechnologies program, MacVector 3.5 (New Haven, CT). The predicted proteins were subjected to database searches against both Genbank and EMBL (Heidelberg, Germany) using BLAST (Altschul *et al.*, 1990), TFASTA (Pearson and Lipman, 1988), and BLOCKS (Henikoff and Henikoff, 1991). The BLAST search was performed at the National Center for Biotechnology Information using the BLAST network service. The protein motif finding programs MacPattern and Motifs were used on both predicted proteins. Motifs is within the GCG Package, Version 7, from the Genetics Computer Group, University of Wisconsin (1991). DNA Strider was used to calculate the predicted molecular weight of the proteins and to determine the amino acid composition. MacVector 3.5 was used to plot hydrophilicity, using the Kyte Doolittle hydrophilicity scale.

Screen for New *lin-15AB* Alleles Starting with a *lin-15B* Allele

Hermaphrodites homozygous for *lin-15B*(n744) were mutagenized at 20°C using ethylmethanesulfonate (EMS) as previously described (Brenner, 1974; Sulston and Hodgkin, 1988). P0 animals were allowed to self-fertilize for two generations, and F2 progeny were screened for Muv animals. Of 6900 mutagenized gametes screened, two new *lin-15AB* alleles, *sy212n744* and *sy211n744*, were recovered.

Noncomplementation Screen Starting with *lin-15AB^{ts}(n765)*

mec-5(e1340) males were mutagenized at 20°C with EMS as above and allowed to mate with *unc-3(e151) lin-15AB^{ts}(n765)* animals. After EMS treatment, all animals and their progeny were grown at 15°C. The F1 generation was screened for Muv animals. Of 35 000 mutagenized gametes screened, no candidates were found. Dominant mutations of other genetic loci were seen, indicating that the mutagen was effective.

unc-3(e151) lin-15AB^{ts}(n765)/mnDf4 animals were constructed by mating N2 males with hermaphrodites of the genotype *mnDp1/+; mnDf4*. *mnDf4* is a deficiency of the X chromosome that removes *lin-15* along with other nearby genes (Meneely and Herman, 1979); this deficiency is covered by the duplication *mnDp1*, which is attached to chromosome V (Herman *et al.*, 1976). This cross was performed at 20°C; the rest of this experiment was performed at 15°C. *mnDp1/+; mnDf4/0* males from this cross were allowed to mate with *dpy-17(e164); unc-3(e151) lin-15AB^{ts}(n765)* hermaphrodites. In the next generation, nonDumpy Uncoordinated hermaphrodites were scored for their Muv phenotype. The nonDumpy Uncoordinated animals were presumed to be of the genotype *dpy-17(e164)/+; unc-3(e151) lin-15AB^{ts}(n765)/mnDf4*. Of the 40 nonDumpy Uncoordinated animals scored, 31 of them (77%) displayed protrusions on their ventral surface that could be easily scored under a dissecting scope.

lin-15A(n767) Deletion Localization

DNA from N2 and *lin-15A(n767)* animals was digested with *EcoRI*, *BamHI*, and *HindIII*, singly and in combination, separated by electrophoresis on agarose gels, and blotted onto Hybond-N according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). These Southern blots were then probed with pgLH2, a 2.8-kb *Sal I/BamHI* fragment containing DNA from base 6886 to 9659 (see Figure 5). The *lin-15A(n767)* deletion was localized to within a *EcoRI/HindIII* 336-bp fragment. Primers were designed to allow for PCR of an ~300-bp fragment from *lin-15A(n767)* DNA. PCR on 50 ng of *lin-15A(n767)* and N2 DNA using both Taq (Cetus) and Pfu (Stratagene) DNA polymerases was performed using primers n767F (GGC GAC CTG CAC AAA GGG AAG GTT ACG TCG) and n767R (CGC TTT CTG CGT TCT ACA GTG TTC TGC ATC). One-tenth of the PCR reaction was separated on a 1% TBE agarose gel, and the PCR product was directly sequenced using primers SQ7F (TTA CGT CGA ACA CTC CGC) and SQ6R (CAG TGT TCT GCA TCT CAC). As primer n767F was only 43 bp away and primer SQ7F was only 33 bp away from the 5' end of the deletion breakpoint, it was difficult to read the sequence on the minus strand all the way to the 5' breakpoint. Therefore, we confirmed the location of both the 5' and 3' breakpoint and the structure of the *lin-15A(n767)* mutation by repeating this procedure using primers n767FZ (TGC CAC CGG ACA GAT TGA TGC GTG GCC ATG) and n767RZ (GGG AAT ATA CGG TTC ACT AGG TGC ATC TTC) for PCR and primers 7SQFZ (GGC CAT GAT ATC TAT AGA GC) and 6SQRZ (GCA TCT TCA GTG TGA TCT CG) for sequencing. Both primer sets revealed the same rearrangement.

lin-15AB(e1763) Deletion Localization

Because of repetitive DNA, we were unable to determine the 3' deletion breakpoint of *lin-15AB(e1763)* by mapping with genomic Southern blots followed by PCR as performed for *lin-15A(n767)*. Therefore, the *lin-15AB(e1763)* deletion was localized using a variation of ligation-mediated PCR (Mueller *et al.*, 1992). Six micrograms DNA from *lin-15AB(e1763)* animals was ethanol precipitated and resuspended in 18 μ l ddH₂O. The DNA was modified by adding 54 μ l 88% formic acid (Mallinckrodt, Paris, KY), vortexing for 25 sec, and incubating for 1 min 30 sec. One hundred sixty-four microliters stop solution (0.36 M NaOAc pH 7.0, 0.14 mM EDTA pH 8.0, 0.5 μ g/ μ l tRNA) was added before ethanol precipitation. The modified DNA was then cleaved by resuspension in 200 μ l 1:10 diluted piperidine (Aldrich, Milwaukee, WI) at room temperature and heating to 90°C for 30 min. Piperidine-cleaved samples were then recovered as described (Mueller *et al.*,

1992). First strand synthesis was carried out on 200 ng cleaved DNA using a denaturing time of 5 min at 95°C, annealing time of 30 min at 50°C, and extension time of 10 min at 76°C. The remainder of the first strand synthesis and ligation were performed as described (Mueller *et al.*, 1992) using primer LMPCR.1 (GCG GTG ACC CGG GAG ATC TGA ATT C), primer LMPCR.2 (GAA TTC AGA TC), and the *lin-15* specific primer, e1763.1 (TCC TTT ATC CAC AAT GGT CA).

The first round of PCR was performed on one-half of the ligated DNA in a mixture consisting of 10 μ l DNA, 20.5 μ l ddH₂O, 2.5 μ l 20 μ M primer LMPCR.1, 2.5 μ l 20 μ M primer e1763.2 (TGT CTA GAA CAC TAT CTC CTC TCC AT), 5 μ l Pfu buffer (Stratagene), 8 μ l 1.25 mM dNTPs, 1.5 μ l Pfu (3.75u, Stratagene), and 50 μ l mineral oil, using PCR conditions as follows: 95°, 1 min; 60°C, 1 min; 72°C, 3 min, 20 cycles total. One-half of the PCR reaction was separated on a 0.8% low melting point TBE agarose gel. DNA migrating between 600 bp and 1.2 kb was isolated from the gel, and one-half of the recovered DNA was subjected to a second round of PCR in a mixture consisting of 10 μ l DNA, 23.5 μ l ddH₂O, 1 μ l 50 μ M kinased primer LMPCR.1, 1 μ l 50 μ M kinased primer e1763.3 (CTA GAA CAC TAT CTC CTC TCC ATT GTC TA), 5 μ l Pfu buffer, 8 μ l 1.25 mM dNTPs, 1.5 μ l Pfu (3.75 u, Stratagene), and 50 μ l mineral oil, using PCR conditions as follows: 95°C, 1 min; 61°C, 1 min; 72°C, 3 min for 25 cycles. One-half of the PCR reaction was separated on a 0.8% low melting point TBE agarose gel, and DNA running between 600 bp and 1.2 kb was recovered from the gel. The recovered DNA was cloned into Bluescript SK⁺ (Stratagene), and four independent isolates were sequenced to determine the 3' breakpoint of *lin-15AB(e1763)*. This result was confirmed by performing PCR on 50 ng of *lin-15AB(e1763)* genomic DNA using primers e1763.chF (GCT GCC ATA AAA TCT AAT AAT CCG CTG CAC) and e1763.chR (TTC TAA CCT GAA AAA TTC CGT GTG ACC GA). The ~350-bp band obtained from this PCR reaction was sequenced to confirm its identity as a *lin-15AB(e1763)* clone.

Construction of *let-23(sy97); lin-15AB(e1763)*

let-23(sy97)/mnC1 (dpy-10 unc-52) males were allowed to mate with *lin-15AB(e1763)* hermaphrodites. L4 hermaphrodite cross-progeny were placed onto individual plates and allowed to self-fertilize. The genotype of *let-23(sy97)/+; lin-15AB(e1763)/+* was determined upon scoring of both Muvs and Vulv in the next generation. Muv and Vul progeny from *let-23(sy97)/+; lin-15AB(e1763)/+* were selected. No Muvs were found segregating from Vul hermaphrodites, whereas Vulv were found segregating from Muv hermaphrodites. The Vulv segregating from the Muv hermaphrodites were the desired genotype *let-23(sy97); lin-15AB(e1763)*.

Gonad Ablations, Vulval Differentiation, Vulval Lineages, and Photography

Animals were mounted on 5% noble agar pads containing ~3 mM sodium azide as an anesthetic, and gonad precursor cells were killed by laser ablation in L1 animals as previously described (Sulston and White, 1980; Avery and Horvitz, 1987; Sternberg, 1988). Ablations were verified 4–20 h after surgery, before the time of vulval differentiation. Vulval differentiation was scored during or after the L3 molt.

To score vulval differentiation, living animals were mounted on 5% noble agar pads and examined under Nomarski microscopy as previously described (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1981). The number of cells undergoing vulval differentiation was tabulated by scoring for the presence of vulval cells versus large nonvulval epidermal cells after the L3 molt, the time after vulval differentiation occurs in intact animals. The percentage of cells undergoing vulval differentiation was determined as previously described (Aroian and Sternberg, 1991). In some cases, vulval cell lineages were followed by watching cell divisions under Nomarski microscopy. Photomicrographs were taken on Kodak technical pan film on a Zeiss Axioskop (Thornwood, NY) under Nomarski optics with a 40 \times objective.

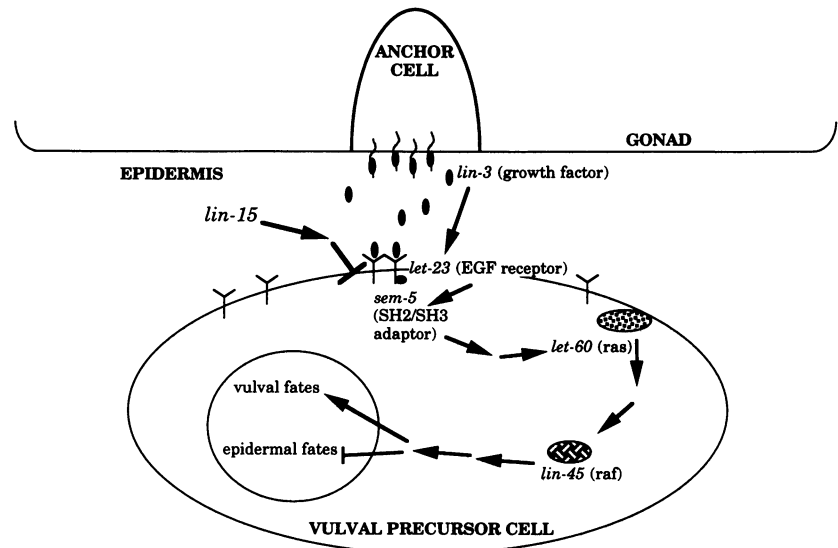


Figure 1. Model for vulval induction. This pathway summarizes the work of many people (see Sternberg, 1993, for review).

RESULTS

Identification of the *lin-15* Genomic Region

We correlated the genetic and physical map in the *lin-15* *sup-10* region of the X chromosome by making recombinants between the highly polymorphic *C. elegans* Berg BO strain and the *C. elegans* Bris strain as described in MATERIALS AND METHODS. By probing the recombinant genomic DNA with radiolabeled probe synthesized from the polymorphism-containing clone, TU#W1723 (M. Driscoll and M. Chalfie, personal communication), we determined that TU#W1723 was approximately one-third of the distance from *sup-10* to *lin-15* (see MATERIALS AND METHODS for details). The cloning of *sdc-1* (Nonet and Meyer, 1991), which genetically maps between *sup-10* and *lin-15* (Villeneuve and Meyer, 1990), confirmed our map data and strongly suggested that *lin-15* was near the end of the then existing *sup-10* contig, an overlapping set of genomic clones for the left end of the X chromosome. Thus, we injected PS#74B3, a cosmid mapping to the end of the

sup-10 contig, overlapping cosmids F14H4 and C29B12 (Mendel and Coulson, personal communication). Microinjection-mediated germline transformation using PS#74B3 rescued the *lin-15* Muv phenotype (Figure 2). To further define the *lin-15* containing region, we injected smaller subclones from the cosmid and found that the pJM23 plasmid would rescue *lin-15*.

Genomic Analysis of the Rescuing Region

Genomic subclones from the region contained in the pJM23 15-kb rescuing fragment were used to probe a Northern blot containing RNA from wild-type animals. Two transcripts were detected (Figure 3). The larger transcript was recognized by both probes (p18B3 and p68B3); the smaller transcript was only recognized by one probe (p68B3). cDNA libraries were probed using a subclone that recognized both transcripts (see MATERIALS AND METHODS). Two noncross-hybridizing classes of cDNAs were found. The clones were mapped to the genomic DNA. As both classes of cDNAs had members that contained poly A⁺ tails, we could infer

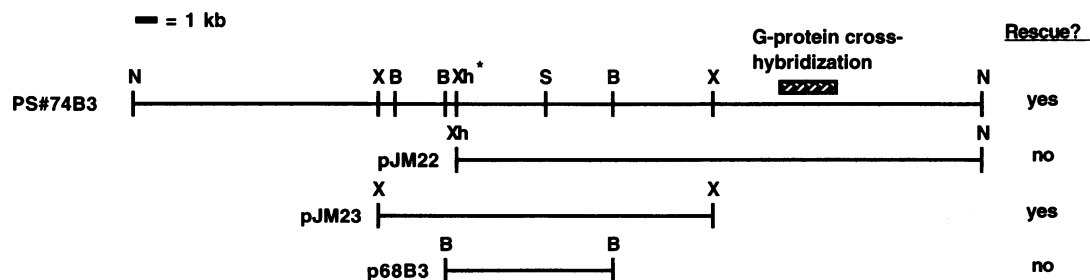
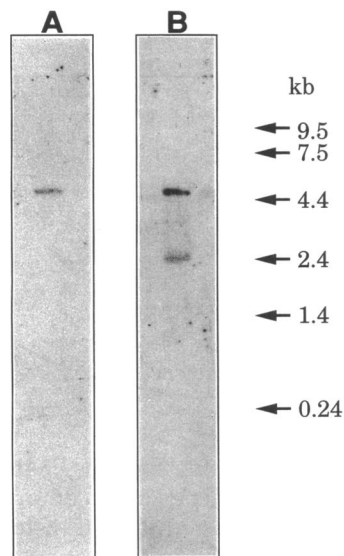


Figure 2. Rescuing ability of cosmid PS#74B3 and various subclones. PS#74B3 was originally isolated as a G-protein cross-hybridizing cosmid (Mendel, personal communication). The G-protein cross-hybridizing region is shown with a hatched box and is distinct from the pJM23-rescuing plasmid. Only relevant restriction sites are shown. Abbreviations: N, Not I; X, Xba I; B, BamHI; S, Sal I. *, two Xho I sites are here, separated by 330 bp. Rescue was demonstrated by microinjection-mediated germline transformation as described in MATERIALS AND METHODS. All injection mixes contained 50 ng/ μ l of the genomic subclone. We injected animals bearing the *lin-15AB*^{ts} allele, *n765*.

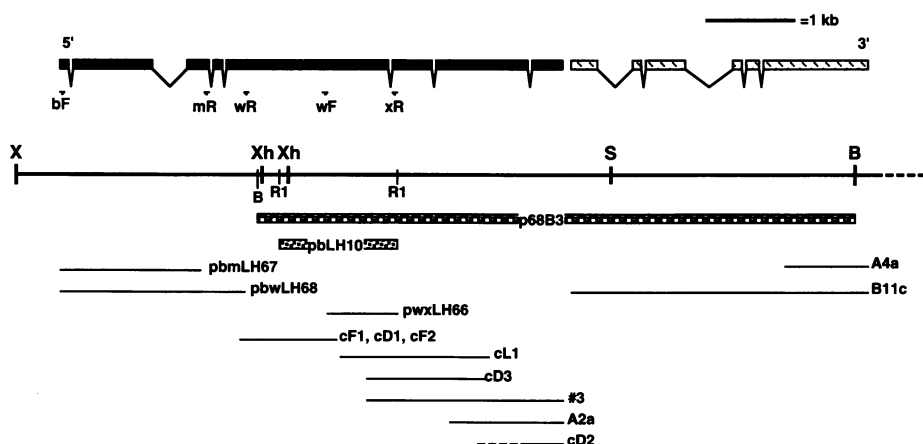


The cDNAs and the corresponding genomic regions were sequenced (Figure 5). Exons are shown in capital letters, and the predicted amino acids are shown below. Only 105 bp of genomic sequence separates the 3' end of the upstream transcript from the 5' end of the downstream transcript.

One cDNA of the downstream transcript, B11c, is likely to be near full length as it contains 3 bp of the *trans*-spliced leader SL2 (Huang and Hirsh, 1989). SL2 is a 22 nucleotide *trans*-spliced leader that is spliced to the 5' end of certain *C. elegans* transcripts (for a review of *trans*-splicing, see Blumenthal and Thomas, 1988). We confirmed that the downstream transcript is indeed *trans*-spliced to SL2 by performing PCR using reverse transcribed cDNA. We were able to amplify a band using the *lin-15* specific primer A4-VR6 and an SL2 primer. This band hybridized to probe made from the B11c cDNA. DNA sequencing confirmed that it was a *lin-15* clone and that *trans*-splicing of SL2 occurs as predicted from the cDNA.

Three cDNA libraries were screened, and a composite cDNA of 3.5 kb was compiled (see MATERIALS AND METHODS and Figure 4). Because this composite cDNA was not complete, introns and exons were predicted from the genomic sequence (see MATERIALS AND METHODS). Predictions were confirmed by performing PCR on reverse-transcribed RNA. From the predicted exons, primers for reverse transcription as well as for PCR were designed. All PCR primers were required to span a predicted intron to allow discrimination of potential genomic contaminants (none were seen). These bands were cloned and sequenced, and the corresponding exons are shown in Figure 4. The first 21 nucleotides of the 5'-most exon, including the initiating methionine, is predicted from the genomic sequence.

Southern blots containing DNA from the *lin-15AB* alleles *n1139*, *n377*, *e1763*, and *n309* were probed with radiolabeled subclones of the 15-kb *lin-15* rescuing ge-



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tctagaacactatctctctccattgtctattttctctctgcccacctgcatagttctgaaccacattgcaagggttccattattccagttctccccccatctctctgaatcgataaggtcc 125
 cgtttgccaacaacogogogcttggccogcaacacogoggttgggtggtgtgogtctccogcogacattcaaatgggttcaacttgctcaogctctctactctctcgttccogcatt 250
 gtatttctctcogcogcagatttgaattttataataaagcacaacattatgtgcttttttctggttaattogagtgacatttttgagttttcagtcaggtaaaagttgtagaattcaaat 375
 tcaoggttttttttctggttaacttttttcaataataatgactttttaaccattctatcatctgttcttagogtattgtatctacogtacttctcgttctttctaca 500
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 AGTGCATgtacgtttattcttgattgataattttctattccaatgcatccaacacattcaacaaacataatttttcagAAGCGACGCAATTCAAAGCGCTTGATCGCGTGAATG 750
 S A I S A A A I Q K T L D A V N R 39
 CCCACGACGCTCAGAGCAAGTGGATTTCTAGCTCAACGTTAOCCTGCCAGCTCCACACAGGACGCTGCCACCATCTTGATGCGGACCCAAAGCAACTGACCTCATGGCTCGCTCTCTTCATCA 875
 P P A V R A S G I L R H R T L P A P T Q E T A H H L D A D P K T T E L M A R F F I S 91
 GCGCAAAATTCGTTGCAATGTGCAACGCGCTTTCTCGGAGCTTTATGAACACGCTGGATTCGAGTGTGATTCACCTTACAAATGTAAACAAAAAATCTGTGGCAAAAATCAGCACA 1000
 Q G I P F E C A H E P A F L E L M K H V D P N C I P P T N V T K K L K I S T 122
 TCATCTAAACCGCAAGTCAATTCACACAAGACTGTGTGGCCACTCAGTGTACCATCGACATTTGTGGAGATGAGCAGCAAAAGTATCTGGCGTTTTCGATTCAITACTTTTGAGATTTGTAGA 1125
 S S K P Q V N Y T K T V G P L S V T I D I C G D E D E K Y L A F S I H Y F E D L Y E 164
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 L 179
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 D S N S L L T N I R R S V N S Y S F S N V K F T N I V C P N E E I C K L V E E S A 220
 GTGTGCAAAAGTACAAAGTTTGTCTTACAACTAGCTTACCGCTTTTGTGGCGGATTTGATGAAMTGAAGAGTTTTCAGTGGGCTGACACAAATTCGCAACATTTGTGTGTTATATGAACA 2250
 V V K R Y N V C F Y N Y V T R F V A D L M E I E E F S S G L T Q L R T F V R Y M K Q 262
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 N S D M Y S K F R R M Q L Q K N A E L D I P S I D S 288
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 G D W H S T A I F L T R C L V W H D T F T E F C 312
 GGAAMTTAGACATCTCCACTACATTCGAAMTGAACATTCACCAACCTTATATACCTCCAGCGCTTCTTCAACATTCGATGAACATTTGTGAGAGCTCAGCATACGCAACATTCGATTTTC 2625
 G K L D I L H Y I D N E T F N H L I Y L Q R L L Q C M K H C R E L S I P N N S I S 354
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 Q V V P A I S N F I A S N S M G Y R F Q K R I R D S F T T S F K E I T S G P S 396
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 Q D R Y D I A T L L D P R F A Y R D T V Y T A Q T W R S L E K K V I D D F V N S D 437
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 M R A A L R L P N S E Q K S G A P S S I N G K V Q R D D F K L E P L D D F N G E P D 896

Figure 5. (Continued)

Figure 5. (Continued)


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R V G D L H K G K V T S N T P L Y S F K N S I K S Y Y R N H V P R M V N G S L S 344
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K P S P K P F S E L V A L L Q S V P P S T N 366
CTAATGAGTTCCTGAATCATATCTTAGCCTCAGTTCCTGATAGCAAGAACTCATTAACATCAATGGGAAAGATAATCGTTTCACATCTCGGAGACGGAAATCGAGGATATCTTGA 8750
L N E L L N H N L S L S D A D K Q E L I Q L I N G K D N R F T S R R R K I E D I L D 408
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N K F A A A A A K A Y R D H S E D A P S E P Y I P N Q S E M Q N T V E R R K R K L H 450
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S P E Q D D A G S S S I S W N A K K T K T P I D Y V H L A T R V L E G H S I A D E 491
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A L L H K S K V S Y A R N A F G E K P S S P T P P S A P L K F C V V N G K K Y L R F 533
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E N G T G P P K V V V Q G N V V L R T N T L K D A L T T A P R A Q N Q P S T S T D S 575
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S S S S E M E G I R Q S F G A P Q K E E E E E E L V P T L L Q N K P T H V E S S S 616
CCCGTGAACAAAAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAAC 9500
P V E K K P P T K T N V E K P A V R L G R M L T T A F S G M S Y R T R K S V E N K T 658
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D L L N Q P T S A S P R R M I K V V R N R N P H L A N K W P P H R V S Q N I F R Q R 700
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T W K R S R K S C S W I Q S L S Q F F K R S * 722
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cggtcacacggaattttttaggttagaaagtggttttgggtgtgtgttttggagctcttttcagaaaaa 9949

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Figure 5. Nucleotide and predicted protein sequence of the *lin-15* locus. The first methionine codon and a potential polyadenylation signal are underlined for each transcript. Uppercase letters indicate exons. The predicted amino acids are shown below. The first exon, part of which is predicted, is shown as starting at a splice consensus 5' to the predicted Methionine. Genomic sequence is shown with base 1 as the *Xba* I site at the 5' end of the rescuing plasmid, pJM23, to 290 bp past the 3' *Bam*HI site at base 9660. EMBL accession number is Z29967.

nomic DNA contained in pJM23. These alleles were all derived from EMS mutagenesis of N2 hermaphrodites (Ferguson and Horvitz, 1985); all exhibited polymorphisms of the *lin-15* rescuing region (Figure 6, lanes 2–5). These polymorphisms were determined to be deletions of the DNA from the *lin-15* rescuing region by mapping of the polymorphisms using *Bam*HI-, *Hind*III-, *Eco*RI-, and *Eco*RV-digested DNAs on Southern blots that were probed with subclones of the 15-kb *lin-15* rescuing genomic DNA contained in pJM23. These deletions appear to affect both transcribed regions.

However, not all Muv alleles of *lin-15* show detectable polymorphisms for the *lin-15* rescuing region. Screens for new *lin-15AB* alleles using EMS starting with the *lin-15B* allele *n744* yielded two new *lin-15AB* alleles, *sy211n744* and *sy212n744*. These alleles were recovered at a frequency of $\sim 1/3500$. We looked for polymorphisms using *Bam*HI-, *Hind*III-, *Eco*RI-, and *Eco*RV-digested DNA on Southern blots that were probed with subclones of the 15 kb *lin-15* rescuing genomic DNA contained in pJM23. No polymorphisms were detected (for example, see lane 13 and 14 of Figure 6). New polymorphisms were also not found for the *lin-15AB* allele *n767sy222*, which was obtained in a similar screen starting with *lin-15A(n767)* (Hill, personal communication) (lane 11).

We were unable to detect polymorphisms associated with the A and B alleles of *lin-15*, except for the *lin-15A* allele, *n767* (lanes 9, 10, and 12 of Figure 6). We determined the location of the *n767* poly-

morphism through restriction analysis followed by PCR. This 179-bp deletion lies in the downstream transcript and spans the final two exons (see Figure 7 for details). The *n767* mutation would lead to a truncated A protein, missing 347 amino acids from the C-terminus, as well as 40 amino acids from the middle of the predicted protein.

All these results together suggest that both transcripts constitute wild-type *lin-15* activity and both must be eliminated to produce a Muv phenotype. This hypothesis is consistent with results from a *lin-15* noncomplementation screen that could recover null alleles, as 77% of animals of the genotype *lin-15AB^{ts}(n765)/Df* are Muv at 15°C. We mutagenized *mec-5(e1340)* males, allowed them to mate with *unc-3(e151) lin-15AB^{ts}(n765)* hermaphrodites, and screened 35 000 F1 progeny raised at 15°C. Although standard mutagenesis protocols result in a *C. elegans* gene knockout frequency between 1/2000 to 1/3300 (Brenner, 1974; Greenwald and Horvitz, 1980; Park and Horvitz, 1986), we recovered no new *lin-15* mutations. However, EMS usually generates point mutations (Anderson and Brenner, 1984; Dibb *et al.*, 1985). Thus, if two independent point mutations or a deletion are required for recovery of *lin-15AB* alleles, mutations that fail to complement *lin-15AB^{ts}(n765)* would be rare. Our failure to recover additional *lin-15* alleles is consistent with *lin-15* being a complex locus.

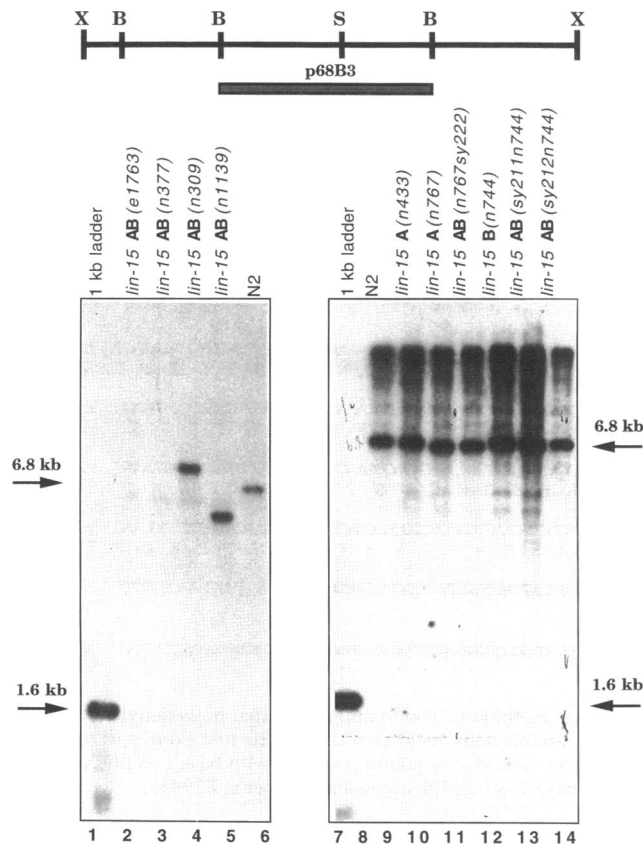


Figure 6. Southern analysis of the *lin-15* locus. One microgram genomic DNA for each strain was digested with *Bam*HI, separated on an agarose gel by electrophoresis, and transferred to a nylon membrane. These membranes were probed with p68B3, a 6.8-kb *Bam*HI genomic subclone. The *lin-15* Muv alleles *e1763*, *n377*, *n309*, and *n1139* are all derived from mutagenesis of the wild-type N2 strain (Ferguson and Horvitz, 1985). The *lin-15* Muv allele *n767sy222* is derived from EMS mutagenesis of *lin-15A(n767)*. The *lin-15* Muv alleles *sy210n744* and *sy211n744* are derived from EMS mutagenesis of *lin-15B(n744)*. Abbreviations: X, *Xba* I; B, *Bam*HI; S, *Sal* I.

The *lin-15* Locus Can Be Divided into a *lin-15A* and a *lin-15B* Region

Because animals carrying either *lin-15A* alleles or *lin-15B* alleles are phenotypically wild-type, it is conceivable that rescued animals could be phenotypically wild-type while still genotypically A or B. To exclude this possibility, we tested for *lin-15A* and *lin-15B* function independently. To test for rescue of *lin-15B*, we injected animals of the genotype *lin-8A(n111); lin-15B(n744)*. To test for rescue of *lin-15A*, we injected animals of the genotype *lin-36B(n766); lin-15A(n767)* (Figure 8 and Table 1). The plasmid pJM24, which contains the genomic sequence that encodes the upstream transcript and the first exon of the downstream transcript, will rescue *lin-8A(n111); lin-15B(n744)* but not *lin-36B(n766); lin-15A(n767)*. Therefore, the upstream transcript is the *lin-15B* transcript.

Transgenes containing pJM23 rescue *lin-36B(n766); lin-15A(n767)* animals to a wild-type vulval phenotype. Therefore, the *lin-15A* region is contained within this plasmid. As pMLH4 does not rescue *lin-36B(n766); lin-15A(n767)* animals, a third transcript 3' of the downstream transcript encoding *lin-15A* function is unlikely. A 12-kb plasmid (pMLH5) containing all of the downstream transcript plus ~3 kb upstream will not rescue *lin-15A*. Although this could indicate a requirement for 5' sequences, we believe this is because transcription of the upstream transcript is required for transcription of the downstream transcript. Spieth *et al.* (1993) have demonstrated that polycistronic transcription exists in *C. elegans* and that polycistronic transcripts are trans-spliced to the SL2 leader. In these polycistronic messages, the downstream transcript is often separated by ~100 bp from the upstream transcript. Because the *lin-15A* transcript is trans-spliced to SL2 and is separated from the upstream *lin-15B* transcript by 105 bp, it is likely that *lin-15A* is being cotranscribed with *lin-15B* and then processed.

The construct pBLH51 is the same as pJM23 except that 330 bp is deleted from an exon in the *lin-15B* transcript. *lin-36B(n766); lin-15A(n767)* animals are rescued to a wild-type phenotype when they carry this construct as a transgene. Injection of pBLH51 into *lin-8A(n111); lin-15B(n744)* animals does not rescue the vulval phenotype. Thus, pBLH51 will rescue *lin-15A* but not *lin-15B*. Because pJM24 will not rescue *lin-36B(n766); lin-15A(n767)* animals, we believe the ability of pBLH51 to rescue *lin-36B(n766); lin-15A(n767)* animals results from the addition of important regulatory sequences for the *lin-15A* transcript, rather than some function of the *lin-15B* gene product.

lin-15A and *lin-15B* Protein Products

The sequences of the predicted protein products from the two transcripts (Figure 5) are different and appear to show no similarity to any currently known proteins. Each transcript contains one large open reading frame that spans all exons. Because the other potential reading frames contain many stop codons and do not code for continuous proteins, we presume the large open reading frames code for the *lin-15A* and *lin-15B* proteins. The methionines at the start of the proteins were chosen because they were the ones closest to the 5' end.

Both predicted proteins are hydrophilic. The 1440 amino acid *lin-15B* protein has a predicted molecular weight of 163 kDa and contains 8.4% acidic and 15.3% basic residues. The 722 amino acid *lin-15A* protein has a predicted molecular weight of 81 kDa and contains 10.7% acidic and 17.8% basic residues. Neither protein contains known protein motifs except for potential N-glycosylation, phosphorylation, and myristoylation sites in both, and potential amidation sites in the *lin-15A* protein. However, the presence of these sites is not suf-

N2 ..G AAC ACT CCG CTT TAC TCA TTC AAG AAT TCC ATT AAA TCT TAT TAC CGT AAC CAC GTG CCA
lin-15(n767) ..G AAC ACT CCG CTT TAC TCA TTC AAG AAT TCC ATT AAA TCT TAT TAC AAA AAA AAA AAA —

N2 AGG ATG GTC AAT GGG TCT CTC AGT AAA CCG TCT CCC AAA CCG TTC TCA GAG CTG GTT GCG CTT
lin-15(n767) —

N2 TTG CAA TCG gtgagtcctgtgacactgtgaaaattttaaaaaatttaaaaaatttcgtttaag GTG CCT CCA TCG
lin-15(n767) —

N2 ACG AAT CTA AAT GAG TTG CTG AAT CAT AAT CTT AGC CTC AGT GAT GCT GAT AAG CAA GAA CTC
lin-15(n767) AAT CAT AAT CTT TAGC CTC AGT GAT ACT GAT AAG CAA GAA CTC

N2 ATT CAA CTC ATC.....
lin-15(n767) ATT CAA CTC ATC.....

Figure 7. The *lin-15A(n767)* mutation. The *lin-15A(n767)* deletion detectable on a Southern blot (see Figure 6) was localized using PCR (see MATERIALS AND METHODS). The sequence in this figure represents the sequence of the PCR fragment obtained by using primer n767FZ and n767RZ as described in MATERIALS AND METHODS. The *lin-15A(n767)* mutation deletes 120 bp from the A transcript as well as a 59-bp intron, while inserting 12 As, thus fusing the final two exons of the *lin-15A* transcript in frame. An extra A is added six bases after the fusion, causing a frame shift resulting in a stop codon three codons later (TGA is boxed). In addition, 11 bp after the stop codon, a G has been changed to an A. The relevance of this G to A change is unknown and could have occurred upon maintenance of this strain, as there would be no pressure to maintain the fidelity of the bases after the new stop codon in *lin-15A(n767)*.

ficient to conclude that these sites are used, and we have no evidence that the presence of these sites is significant.

e1763 Is a Candidate Null Allele of *lin-15*

As Southern blot analysis demonstrated *lin-15AB(e1763)* was a deletion of a large part of the genomic DNA contained in pJM23, we localized the deletion breakpoints of *lin-15AB(e1763)* by ligation-mediated PCR. The extent of the deletion was confirmed by PCR directly from *lin-15AB(e1763)* genomic DNA. Our analysis demonstrates that the *e1763* allele deletes DNA from base 33 beyond the *Xba* I site to base 9588 in the 3' UTR of the *lin-15A* transcript, effectively deleting all coding regions contained in the rescuing fragment, pJM23. Previously

published experiments with *lin-15* have used the allele *n309*, which contains a smaller deletion than *e1763* (see Figure 8). Thus, we characterized animals carrying the *lin-15* molecular null, *e1763*, to determine if there were any differences that would affect interpretation of earlier studies.

Gonad Independence of *lin-15AB(e1763)* Vulval Differentiation

In *lin-15AB(e1763)* animals, essentially all VPCs undergo vulval differentiation. To determine if the Muv phenotype of *lin-15AB(e1763)* mutant animals is dependent on the inductive signal from the gonad, we performed gonad ablation experiments. *lin-15AB(e1763)* animals whose gonads were ablated in the first larval stage (L1)

Figure 8. The *lin-15* locus can be separated into a *lin-15A* and a *lin-15B* region. The orientation of the two transcripts of the *lin-15* locus is shown on the top left with the intron/exon structure of the two transcripts below a partial restriction map of the 15-kb *Xba* I subclone, pJM23. The location of the deletions for the *lin-15AB* alleles, *e1763* and *n309*, and the *lin-15A* allele, *n767*, are shown above. Abbreviations: X, *Xba* I; Xh, *Xho* I; R1, *Eco*RI; S, *Sal* I. Rescuing ability was determined using germline transformation (see MATERIALS AND METHODS). The *e1763* deletion was localized as described in MATERIALS AND METHODS; the deletion is described in the RESULTS. The *n309* defect was localized using Southern blots probed with different regions of the *lin-15* genomic region. The left breakpoint lies somewhere between the second *Xho* I site and the *Eco*RI site shown. The right breakpoint is either close to the *Bam*HI site or to the right of it; we were unable to determine its location because of repetitive DNA to the left of the *Bam*HI site. The *n767* defect is described in Figure 7. To determine the ability of a genomic subclone to rescue *lin-15B*, animals of the genotype *lin-8A(n111)*; *lin-15B(n744)* were injected. To determine the ability of a genomic subclone to rescue *lin-15A*, animals of the genotype *lin-36B(n766)*; *lin-15A(n767)* were injected. See Table 1 for details.

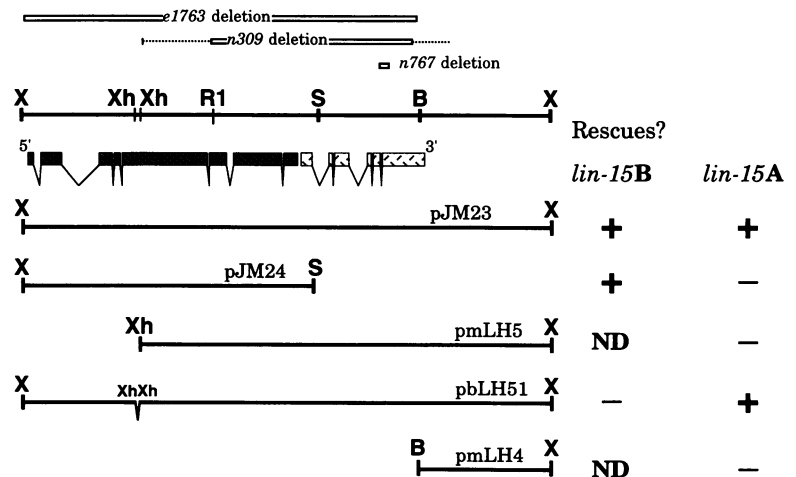


Table 1. Rescue of *lin-15A* and *lin-15B*^a

Plasmid injected	Concentration injected	Rescue of <i>lin-36B</i> (n766); <i>lin-15A</i> (n767)				Rescue of <i>lin-8A</i> (n111); <i>lin-15B</i> (n744)			
		Number F1 rescued	Total number F1s	Number stable lines rescued	Number of stable lines	Number F1 rescued	Total number F1s	Number stable lines rescued	Number of stable lines
pJM23	50 ng/μl	8 ^d	8	4	4 ^e	0	16	1	1 ^f
pJM23	25 ng/μl	29	29	3	3	5	24	4	4 ^g
pbLH98 ^b	25 ng/μl			ND		33	50	11	11
pbLH99 ^c	25 ng/μl			ND		26	42	8	8
pJM24	50 ng/μl	0	11	0	3	15	16	2	2
pmLH5	25 ng/μl	0	15	0	2			ND	
pbLH51	50 ng/μl	27	28	5	5	0	19	0	1
pbLH51	25 ng/μl			ND		0	26	0	2
pmLH4	25 ng/μl	0	27	0	5			ND	

^a Stable rescue of *lin-15A* and *lin-15B* was determined by transmission of the Rol phenotype, unless otherwise noted. These Rol nonMuv animals typically have progeny rescued for *lin-15*, which are nonMuv Rol and nonMuv nonRol (which are rescue for *lin-15* but do not express the Rol marker), as well as nonrescued progeny, which are Muv nonRol. This is a typical range of phenotypes, as injected DNA exist in extrachromosomal arrays that are not transmitted to 100% of an animal's progeny.

^b pbLH98 is an independently derived isolate of the 15-kb *lin-15*-rescuing fragment subcloned directly from cosmid PS#74B3, like pJM23. pbLH98 and pJM23 carry the insert in the *Xba* I site of Bluescript SK⁺ (Stratagene) in the same orientation.

^c pbLH99 is an independent isolate of the 15-kb *lin-15*-rescuing fragment subcloned directly from cosmid PS#74B3 into the *Xba* site of Bluescript SK⁺ (Stratagene) in the opposite orientation of pJM23 and pbLH98. We cannot explain the incomplete F1 rescue of pJM23, pbLH98, and pbLH99 of *lin-15B*, especially given the rescuing ability of pJM24 of *lin-15B*. However, this incomplete rescue does not appear to be caused by a mutation in pJM23 or by the orientation of the insert relative to the vector sequences.

^d Of the eight F1 rescued animals, only two of the eight were nonMuv Rol; the other six were nonMuv nonRol.

^e Of the two Rol F1-rescued lines, both produced Muv and nonMuv progeny, although the Rol phenotype used as a selectable marker was lost. Of the six nonMuv nonRol F1 animals, two of the six produced both Muv and nonMuv progeny, although no Rol animals were seen. This suggests that the expression of the pRF4 Rol plasmid was not sufficient for cotransformation with pJM23. Thus, we lowered the concentration of pJM23 to 25 ng/μl and the results are given above.

^f This stable line segregated nonMuv Rol, Muv Rol, nonRol Muv, and nonRol nonMuv animals.

^g One stable line never segregated Rol animals but segregated animals that were nonMuv. When these nonMuv nonRol animals were cloned, they segregated Muv and nonMuv animals, but no Rol animals. The other stable lines behaved normally.

display vulval differentiation (see Table 2). This gonad independence is consistent with previous results using *lin-15AB*(n309) (Sternberg and Horvitz, 1989).

Epistasis of *lin-15AB*(e1763) and *let-23*(sy97)

To confirm the order of action of *lin-15* and *let-23*, the candidate receptor for the inductive signal, we performed double mutant analysis with *let-23*(sy97), the most severe viable allele of *let-23* (Aroian and Sternberg, 1991). Wild-type animals are defined as having 100% vulval differentiation, corresponding to three of six VPCs generating vulval tissue (Figure 9 and Table 2). Animals homozygous for *let-23*(sy97) are Vul and display no (0%) vulval differentiation; animals homozygous for *lin-15AB*(e1763) are Muv and have essentially 200% vulval differentiation (all six VPCs assume vulval fates). The double mutant *let-23*(sy97); *lin-15AB*(e1763) displays the *let-23*(sy97) Vul phenotype and 14% vulval differentiation. Gonad ablations on *let-23*(sy97); *lin-15AB*(e1763) animals were performed to determine whether the residual activity is gonad dependent. No vulval differentiation was observed in gonad ablated

let-23(sy97); *lin-15AB*(e1763) animals. Therefore, the residual differentiation seen in *let-23*(sy97); *lin-15AB*(e1763) animals is probably because of residual activity of the sy97 allele (see below).

DISCUSSION

We have cloned the *C. elegans lin-15* locus by isolating a 15-kb genomic fragment, pJM23, that will rescue the *lin-15* Muv, A, and B phenotypes in transgenic animals. *lin-15AB* alleles n309, n1139, n377, and e1763, derived from EMS mutagenesis of a wild-type strain, all contain deletions corresponding to sequences within this rescuing fragment. Two transcripts are encoded by this region, transcribed in the same direction, and separated by 105 bp. The upstream transcript encodes the *lin-15B* activity; a subclone containing essentially only the upstream transcript will rescue *lin-15B* mutant animals but not *lin-15A* mutant animals. Two experiments suggest that the downstream transcript encodes the *lin-15A* activity. First, the *lin-15A* allele n767 contains a deletion/insertion in the downstream transcript. Second, pbLH51, a derivative of pJM23 that deletes 330 residues from an

exon of the upstream B transcript, will rescue *lin-15A* but not *lin-15B*. Clark, Lu, and Horvitz (personal communication) have also cloned *lin-15* and have obtained similar results.

lin-15A and lin-15B Encode Novel Gene Products

Sequence analysis of the cDNAs and genomic sequence corresponding to the two *lin-15* transcripts indicates their predicted protein products are not similar to any currently identified proteins. Furthermore, motif analyses show no significant recognizable protein motifs. Because the *lin-15* locus behaves nonautonomously in genetic mosaic analysis, it has been proposed that *lin-15* is or controls the production of a negative signal produced by the epidermis that prevents vulval differentiation (Herman and Hedgecock, 1990). Because there are no clear consensus signal sequences, it is unlikely that either *lin-15A* or *lin-15B* is secreted. Thus, *lin-15* may regulate production of such a signal. However, the lack of a signal sequence does not formally rule out the possibility that either product of *lin-15* may be secreted. For instance, *Saccharomyces cerevisiae* α -factor is clearly exported although its sequence does not have a consensus signal sequence (Brake *et al.*, 1985).

It is not surprising that *lin-15A* and *lin-15B* are novel gene products, because they act in a pathway that nonautonomously regulates RTK activity or effect, originating from neither the signaling nor the receiving tissue but from a third tissue, the nonvulval epidermis. Such nonautonomous regulators of RTKs have not been previously identified because of the assays typically used to study RTKs. Cell culture systems usually contain homogeneous cell populations supplied with growth factors in the media; in vivo the receiving cell resides near the signaling tissue and is surrounded by other cell types. Therefore, a negative signal from a nonreceiving nonsignaling tissue might not have been detected by cell culture assays. A genetically identified locus in *Drosophila*, *argo/giant lens(gil)*, may behave like *lin-15* in *Drosophila* photoreceptor development (Freeman *et al.*, 1992; Kretzschmar *et al.*, 1992); *argos/gil* acts nonautonomously to affect cell fate determination (Freeman *et al.*, 1992) and encodes a potentially secreted factor with an EGF-like motif that displays no sequence similarity to either *lin-15A* or *lin-15B*.

What Is the Nature of the Redundancy of lin-15A and lin-15B?

Two genetic activities, A and B, were previously defined for *lin-15* as well as for the other A and B genes, such as *lin-8A*, *lin-9B*, *lin-36B*, *lin-37B*, and *lin-38A* (Ferguson and Horvitz, 1989). Although *lin-15* was shown to have both A and B genetic activity, the mechanism of this dual function was unclear. For instance, the *lin-15* locus could have encoded a bifunctional protein that could be independently mutated to become defective in either

A or B activity. This would have suggested that *lin-15* was an integrator of the A and B pathways. Instead, the *lin-15* locus contains two nonoverlapping transcripts; one encodes A function, the other encodes B function. In fact, the *lin-15* locus could be viewed as containing a class A and a class B gene. Their physical juxtaposition could have no more functional significance than, for example, if *lin-8A* and *lin-9B* were to be tightly linked on the same chromosome. However, the *lin-15* genomic structure fits the emerging consensus for polycistronic transcripts (Spieth *et al.*, 1993). Thus, it is possible that *lin-15A* and *lin-15B* together are important either in controlling or in being controlled by other A and B genes because of the potential for *lin-15A* and *lin-15B* to be cotranscribed and coordinately regulated.

Ferguson and Horvitz (1989) demonstrated that A and B activities are redundant, because animals homozygous mutant for either A or B are wild-type; animals display the Muv phenotype only when homozygous mutant for both A and B. However, the nature of the redundancy was unknown. Our transgenic studies demonstrate that the *lin-15A* product and the *lin-15B* product cannot substitute for each other, nor can they substitute for other class A and class B genes. If *lin-15B* could substitute for *lin-15A*, we would not have been able to distinguish the two transcripts by rescue experiments, as injection of a subclone containing the *lin-15B* transcript would have rescued *lin-15A* and vice versa. Furthermore, if *lin-15B* could substitute for *lin-36B*, injection of a subclone containing the *lin-15B* transcript would have rescued *lin-36B*. A similar argument can be used for *lin-15A* substituting for *lin-8A*. Finally, sequence comparison of the *lin-15A* protein to the *lin-15B* protein reveals no similarity. Therefore, A and B are genetically redundant but not molecularly redundant. They do not appear to encode related proteins and are not able to functionally substitute for each other in transgenic assays.

Role of lin-15 in Regulation of the let-23 Pathway

lin-15 acts as a negative regulator of the *let-23*-mediated signaling pathway (Ferguson *et al.*, 1987). Our genetic analysis demonstrates that *lin-15* acts upstream of *let-23*; a *let-23* Vul mutation is epistatic to a *lin-15* Muv mutation. This conclusion is supported by the fact that alleles of *let-23*, as well as alleles of the downstream genes *sem-5*, *let-60*, and *lin-45*, have been isolated as suppressors of *lin-15* (Beitel *et al.*, 1990; Han *et al.*, 1990, 1993; Clark *et al.*, 1992a,b). Furthermore, the patterning of vulval differentiation in *lin-15* mutant animals is dependent on the inductive signal. P6.p, the VPC closest to the AC (Sulston and Horvitz, 1977), always adopts a 1° fate in intact *lin-15* mutant animals, whereas in gonad-ablated *lin-15* mutant animals, P6.p can adopt either a 1° fate or a 2° fate (Sternberg, 1988). VPCs in *lin-15* mutant animals thus retain the ability to respond

Table 2. Double mutant analysis of *lin-15* and *let-23*

Genotype	Gonad	Percentage ^a differentiation	n ^b	# of VPCs differentiated ^c					
				0	1/2, 1	1 1/2, 2	2 1/2, 3	3 1/2–5	5 1/2, 6
N2	+	100	many ^d				all		
N2	–	0	many ^e	all					
<i>lin-15(e1763)</i>	+	199	28						28
<i>lin-15(e1763)</i>	–	200	13						13 ^f
<i>let-23(sy97)</i>	+	0	19	19					
<i>let-23(sy97)</i>	–	0	3	3					
<i>let-23(sy97); lin-15(e1763)</i>	+	14	40	24	13	2	1		
<i>let-23(sy97); lin-15(e1763)</i>	–	0	15	15					

^a Percentage differentiation reflects the amount of VPC differentiation observed of a genotype relative to wild-type animals.

^b n is the number of animals examined.

^c The numbers in this column represent the number of animals displaying a particular amount of VPCs differentiated.

^d See Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986.

^e See Sulston and White, 1980; Kimble, 1981.

^f Occasionally, P cells were killed during gonad ablations of *lin-15AB(e1763)*, resulting in fewer than normal VPCs. However, all VPCs present differentiated in the *lin-15AB(e1763)* animals.

to inductive signal. Therefore, *lin-15* acts in parallel with the inductive signal to regulate *let-23*. Because we use vulval differentiation as the assay for activity of *let-23* and *lin-15*, we can only discuss the genetic activities of these loci; our analysis does not imply a direct biochemical interaction of *lin-15* with *let-23*. Formally, the *let-23* pathway could act positively on a downstream molecule, X, whereas the *lin-15* pathway acts negatively on X in parallel to the *let-23* pathway (where X has no activity completely dependent on ligand stimulated *let-23*). Our genetic analysis indicates that *lin-15* regulates

let-23, as *lin-15* is required only in the presence of functional *let-23*.

It is clear that *lin-15* regulates *let-23* basal activity. Vulval differentiation occurs independently of the inductive signal in *lin-15AB(n309)* mutant animals (Ferguson *et al.*, 1987; Sternberg and Horvitz, 1989). Similarly, in *lin-15AB(e1763)*, a molecular null, all VPCs undergo the cell division patterns and morphogenetic movements characteristic of vulval fates even in the absence of the gonad. The VPC's ability to undergo inductive-signal independent differentiation implies *lin-*

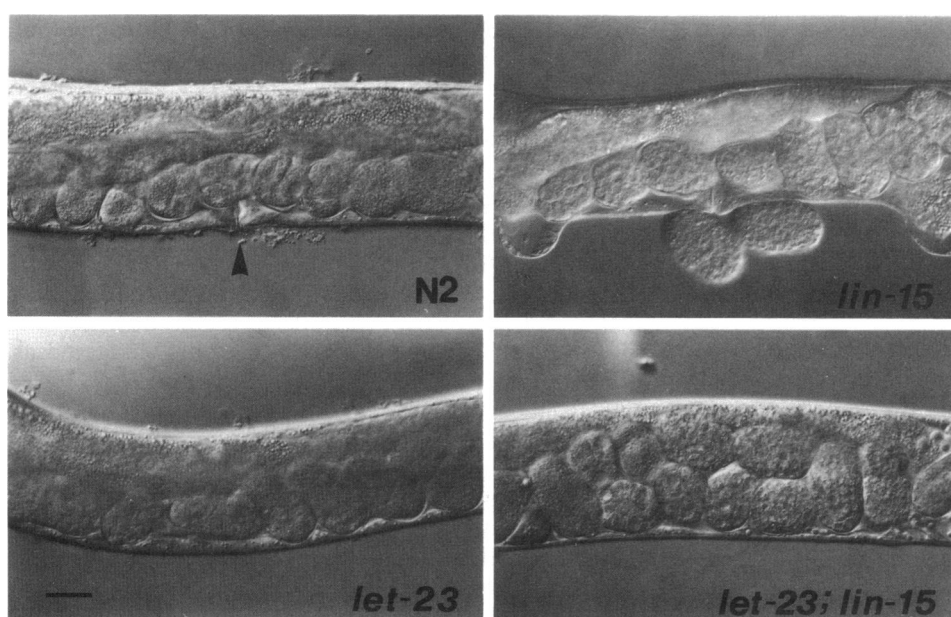


Figure 9. Double mutant analysis of *let-23(sy97); lin-15AB(e1763)*. All photographs are of the middle third of adult hermaphrodite animals. Top left: the wild-type N2 strain. Arrow points to vulva. Top right: *lin-15AB(e1763)*. Bottom left: *let-23(sy97)*. Note the lack of a functional vulva. Bottom right: *let-23(sy97); lin-15AB(e1763)*. Bar, 20 μ m. See text.

15 regulates *let-23* basal activity, where basal activity is defined as the potential of *let-23* activity to cause vulval differentiation in the absence of its ligand.

We cannot conclude whether *lin-15* preferentially regulates basal or stimulated activity of *let-23*. Signal-dependent vulval differentiation is seen in the *let-23(sy97); lin-15AB(e1763)* double mutant, although the extent of vulval differentiation is not wild-type. Thus, removal of the negative regulation conferred by *lin-15* allows detection of the low level of residual-stimulated activity of the LET-23(sy97) mutant protein. The response of the LET-23(sy97) mutant protein to ligand activation is not surprising, as the *let-23(sy97)* allele harbors a point mutation in a 3' splice acceptor, causing misspliced RNAs that, when translated, would result in a C-terminal truncation of the protein (Aroian *et al.*, 1994). Because this truncation does not disrupt the kinase domain and *let-23(sy97)* can function in at least one tissue (Aroian and Sternberg, 1991), the truncated molecule retains a low level of activity. The gonad-dependent differentiation seen in *let-23(sy97); lin-15AB(e1763)* implies that *lin-15* has an effect on ligand bound receptors, assuming that all receptors present were occupied by ligand during the signaling event. However, if receptors are in excess on each cell, it would be unclear whether *lin-15* activity regulates both ligand unbound as well as ligand bound receptors.

Negative Regulation and Inductive Signaling

The signal transduction pathway functioning in vulval development uses *C. elegans* homologues of molecules involved in cell proliferation and determination throughout the animal kingdom. Besides *lin-3* and *let-23*, there is *sem-5*, SH2/SH3 adaptor protein, *let-60*, a *ras* protein, and *lin-45*, a *raf* protein. This particular signaling cascade is used in *Drosophila* R7 photoreceptor determination and in the proliferative response of mammalian cell culture systems. The high degree of conservation of these components throughout the animal kingdom strongly suggests that *lin-15* homologues may exist in these and other systems.

The emerging picture of signal transduction shows that, in general, receptors are expressed with less specificity than their ligands. For example, the RNA and protein products of the *Drosophila* PDGF-family RTK *torso* have no apparent localization and are expressed all over the embryo, even though *torso* acts to specify cell fate in the termini of the embryo (Sprenger and Nüsslein-Volhard, 1992). Similarly, the RTK *sevenless* required for *Drosophila* R7 photoreceptor development is expressed in cells other than R7 (Banerjee *et al.*, 1987; Tomlinson *et al.*, 1987). The localization of the *boss* ligand to the apical surface of R8, the signaling cell, is one reason why R7 is the only *sevenless* expressing cell that responds to the *boss* ligand (Van Vactor *et al.*, 1991).

During *C. elegans* vulval development all six VPCs are competent to undergo vulval differentiation upon

receipt of the inductive signal (Sulston and White, 1980; Sternberg and Horvitz, 1986; Thomas *et al.*, 1990; Hill, Katz, and Sternberg, unpublished data). Presumed overexpression of the *lin-3* signal causes up to six VPCs to adopt vulval fates (Hill and Sternberg, 1992). Thus, *lin-3* must be precisely regulated during wild-type vulval development.

As Gurdon (1992) points out, the number of cells competent to respond to a particular signal exceeds the number that actually respond. Davidson (1993) has argued that competence could be viewed as a cell possessing the appropriate receptors, signal transduction molecules, and transcription factors, with the molecular response of a cell being limited by the expression of the signal. Receptor tyrosine kinase activation most likely involves precise temporal, spatial, and quantitative control of ligand expression. However, because receptor is more ubiquitously expressed, receptor activity must be precisely regulated to prevent incorrect development. Thus, the study of *lin-15* and how this pathway prevents inappropriate RTK activity should yield new insights on RTK regulation as well as how cells can respond appropriately to a localized ligand.

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